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Freeze Injury of the Tibialis Anterior Muscle

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Abstract

Freeze injury is physically induced by exposing skeletal muscle to an extremely cold probe, and results in a robust degenerative and inflammatory response. One unique aspect of freeze injury is that it destroys not only the muscle fiber cells, but also all of the mononuclear cells in the zone of injury. Repair of the muscle is accomplished by satellite cells from outside of the zone of injury, which must migrate in and which may interact with inflammatory cells, hence the length of time before apparent histological recovery of the most damaged zone is typically somewhat longer with freeze injury than with other physical or chemical methods of injury. In this chapter, we present a detailed protocol for the freeze injury of the tibialis anterior (TA) muscle in mouse.

Keywords

Freeze injury; Tibialis anterior muscle; Regeneration

1 Introduction

Traumatic skeletal muscle injuries, such as those induced by extreme cold, crushing, or toxins, result in profound pathological changes and loss of muscle function, which require greater than 2 weeks to recover [1–3]. The regenerative potential of skeletal muscle under such experimental conditions is classically measured by inducing a defined amount of damage to muscle, waiting for a defined period of time, then monitoring muscle repair either histologically or with functional measurements. Many cell types contribute to the regeneration and repair of skeletal muscle, including the initial inflammatory infiltrate [4] the resident mesenchymal cell population [5–7] and of course satellite cells, the stem cells for skeletal muscle [8–12]. Inflammatory cells may directly influence differentiation, as coculture of myogenic cells with macrophages enhances formation of differentiated myotubes [13, 14]. Pro-inflammatory cytokines such as TNF- α and the chemokine receptor CCR2 are certainly necessary for complete and rapid regeneration of injured skeletal muscle [15–18]. Recent work, however, suggests that the inflammatory response orchestrates a flux in the activity of the mesenchymal cell population, which regulates the proliferation and differentiation of the activated satellite cells [19, 20].

Many commonly used injury models focus destruction on the contractile functional unit of skeletal muscle, the myofiber. These include agents such as BaCl and cardiotoxin that cause hypercontraction and death of fibers, physiological “overuse” injuries driven by electrically forced contraction, and physical crush injury, which disrupts the integrity of extremely large

cells such as fibers, and disrupts the integrity of tissue components such as the vascular bed, but leaves mononuclear cells, for the most part, alive. An interesting counterpoint to these types of injury is the freeze injury, in which the rapid freeze–thaw kills all cells in the zone of injury, thus the repair process requires migration in of both the nonmyogenic support population as well as the myogenic progenitor cell population.

Studies in which an entire rat extensor digitorum longus (EDL) muscle was frozen in situ have suggested that myogenic progenitors cannot migrate in from adjacent muscles, nor can they transit through the circulation [21]. Grafts of freeze-killed EDL muscle sutured into the space left by removal of the TA and EDL can be repopulated by third party myogenic progenitor cells (injected into the frozen donor muscle at the time of grafting), which then form new muscle distinguished from the host by glucose-6-phosphate isomerase polymorphism [22]. Interestingly, this experiment provided evidence in favor of the possibility of immigration of myogenic progenitor cells, in the form of hybrid isoenzymes detected in some recipients.

Profiling of freeze injury compared to contraction-induced injury demonstrated a much greater inflammatory response [23]. In the protocol described below, a large zone in the belly of the TA muscle is killed, but the ends of the muscle are spared. Thus myogenic progenitors from the spared zones of the same TA muscle being frozen are largely responsible for its regeneration. As an inflammation-predominant muscle injury, freeze injury is an ideal model for research related to inflammatory response or inflammatory cell-driven regeneration.

The freeze injury protocol described below is ideally performed by a team of 3: an individual to prepare the animal for the procedure, a freeze-injury surgeon, and a suturing surgeon. This is especially convenient when a large number of animals are to be injured.

2 Materials

1. Isoflurane units (3× if possible).
2. Anesthesia tubing and nose cone.
3. Polycarbonate square.
4. O₂ tanks (3×).
5. Recirculating heating pad.
6. Dissecting microscope.
7. Hot bead sterilizer.
8. Isoflurane.
9. Buprenorphine.
10. Kimwipes.
11. Saline.

12. Artificial tears.
13. Nair hair remover.
14. Cotton-tipped swab.
15. Betadine solution and scrub.
16. Cotton swabs.
17. 70 % alcohol.
18. Fine surgical scissors.
19. Fine tip forceps ($\times 2$).
20. Suture tool.
21. Dry ice.
22. Steel freeze probe; for mouse TA muscle a 6-mm-diameter cylindrical rod works well (Fig. 1).
23. 6-0 silk sutures with needles.
24. Food pellets.
25. Small petri dish.
26. Hydrogen peroxide (H_2O_2).
27. Vetbond or superglue.
28. Distilled water.
29. Square steel platform for foot support.
30. Duct tape.
31. 0.5 mL or 1.0 mL syringe.
32. New Skin liquid bandage.
33. Metronidazole.
34. Surgical gloves.
35. Timer.

3 Methods

The procedures described below are for the mouse TA muscle, but can be adapted for other muscles, or for the rat.

3.1 Preparation

1. Weigh the mouse.
2. Attach tubing from isoflurane vaporizer to the anesthesia induction chamber and set the isoflurane concentration to 5 % and the O_2 gas flow rate to 1000 mL/min.

3. Place mouse in chamber until the mouse stops moving and breathing has slowed to 1–2 breaths per second.
4. Attach the anesthesia tubing to the nose cone and change isoflurane concentration to 2 % and the gas flow rate to 200 mL/min.
5. Place mouse in the supine position on the clean Kimwipes with its nose just inside the nose cone.
6. Apply artificial tears to the eyes of the mouse.
7. Shave hair from the lateral and medial aspects of the left/right/both hindlimb(s). Shave up to the quadriceps about 3 mm above the knee and all the way down to the ankle.
8. Apply Nair to shaved areas with a cotton-tipped swab and leave on for ~2 min.
9. Remove Nair with Kimwipes and clean off the remaining Nair with distilled water.
10. Prepare the aseptic skin by applying Betadine in a circular pattern moving outward from the center of each shaved area three times, using a new, clean Q-tip with each new application.
11. Wipe off Betadine by applying cotton swab with 70 % alcohol. Make sure each stroke of the cotton swab is with a new, clean swab. Try not to get the mouse's coat too wet with alcohol when cleaning.
12. If 3 stations are available, move the mouse to the surgical station. Make sure to clean nose cone and replace with clean Kimwipes between each mouse in this preparation station.

3.2 Injury

1. In surgical area, turn the gas flow rate to 100–200 mL/min and set the isoflurane concentration to 1–2 %. Do this 2–3 min ahead of time; this is especially important for the first mouse of the day.
2. To injure the left TA muscle, place mouse in right lateral recumbency, secure the left hind foot, ideally by placing it on a 30 mm high block (*see* Note 1) and secure the proximal end of the tail with tape. If both hindlimbs are required, perform one side and then turn to the other side.
3. The surgeon should clean hands and lower arms with Betadine scrub thoroughly and put on surgical gloves to avoid contamination. During the injury, the surgeon is required to maintain an aseptic environment until end of process.
4. Make incision through the skin on the left hind limb to expose the TA muscle (Fig. 2a). This first incision is small (i.e., ~2 mm) and over the patellar tendon

¹This is important! The goal is for the lower leg to be parallel with the table so that when the probe is applied it is done so “flat” to produce an equal-force injury.

- but perpendicular to the tendon. Use fine forceps and scissor to make the incision.
5. Make a second incision continuing from the first incision but running perpendicular, i.e., over the lateral edge of the TA down to the ankle. Use fine scissors to make the incision while using fine tip forceps to hold the skin during the incision.
 6. Use two pairs of fine tip forceps to loosen the skin from the underlying muscle.
 7. Grab behind the knee with your left hand to support the left leg of the mouse.
 8. Take the steel probe that has been precooled in dry ice (at least 30 min) and hold as if you were holding a pencil. Place the probe tip on the mid-belly of the TA (the fattest part of the muscle, Fig. 2b) and press firmly for exactly 10 s (*see Note 2*). The degree of injury from this freezing protocol is very large (Fig. 3), and muscle takes about 3 weeks to return to normal strength (Fig. 4).
 9. Wait for ~1 min for the freeze-injured area to change from its frosted color back to the normal pink color, then apply 1–2 drops of saline.
 10. Under a dissecting microscope: suture the skin incision using 6-0 silk suture. To start, ensure that the foot is taped down so the TA is fully exposed and stretched out so that it is possible to suture up the entire incision. Start suturing at the ankle. Make 4–5 throws per knot. Cut the suture as close to the knot as possible. Make sure there are no visible gaps in the skin.
 11. Use a cotton tipped swab to clean the sutured incision with H₂O₂. Dry the area and place Vetbond or superglue on the sutured incision (*see Note 3*). Clean off excess glue with a cotton-tipped swab.
 12. Wait until the Vetbond or superglue dries completely and then apply a mixture of New Skin and metronidazole by drawing a fine line to cover the sutures.
 13. Sanitize tools in hot bead sterilizer and clean area with 70 % alcohol in between each mouse.
 14. Give each mouse 0.1333 mg/kg subcutaneous buprenorphine injection. Place mouse in cage and place cage on a heating pad set to low (*see Note 4*).
 15. Wait until the mouse is alert and moving around cage before returning the mouse to its housing room (*see Note 5*).
 16. Soak food pellets in water for ~1 min and place four softened pellets in a small petri dish with additional water inside cage with the mouse. Make sure water bottle in the cage is positioned correctly (*see Note 6*).

²Set a timer. Keeping the probe in contact for too long can lead to freezing of the adjacent skin. This must be carefully avoided, to avoid infection and necrosis.

³To avoid skin stiffness post-surgery, use as little glue as possible—control the flow of glue to minimize the amount of Vetbond or superglue on the incision site.

⁴Take care not to set the heating pad too high as this is a common way to harm the mouse.

⁵Mice can be group-housed after surgery.

3.3 Cleanup

1. Turn off isoflurane, heating pad, and O₂ tanks.
2. Soak all surgical instruments in warm, soapy water for 4–5 min.
3. Clean instruments with steel brush. Pat dry, place in autoclave bags and seal. Place autoclave indicator tape on bag and label with name and date. Autoclave for 45 min at 121 °C followed by a 10-min exhaust.
4. Soak anesthesia induction chamber and weighing container in warm soapy water for 4–5 min, rinse, dry and return to counter.
5. Clean recirculating heating pad, underlying surface, nose cone, and counter with 70 % alcohol.

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⁶Watch mice closely for the first 3-days post-injury (at least twice per day). In case signs of infection appear or the incision site reopens, take action immediately.

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Fig. 1. Steel probe used to induce freeze injury. *Top:* A probe of length 25 mm is fixed to a 6-mm steel rod covered by autoclavable PVC (~14 mm outer diameter cylinder to the right). *Bottom:* Enlarged end of the freeze probe showing the shape of the tip

Fig. 2.

Exposure of the TA muscle and positioning of the leg. *Left:* TA muscle is exposed by incision of the skin. *Right:* The probe, previously buried in dry ice for at least 30 min, is applied to the TA muscle without touching the skin. Note that the leg is positioned such that the surface of the TA muscle is horizontal so that when the probe is applied vertically the pressure is uniform

Fig. 3.

Representative images of hematoxylin and eosin-stained cross sections of frozen mouse TA muscles. In both images the anterior surface of the muscle, that is, the freeze-injured portion, is at the top. *Left:* TA muscle 1 day post freeze injury. About 75 % of the muscle cross section is damaged. Compared to fibers in the uninjured region, fibers in the damaged region have lost their normal polygonal shape. There is a modest influx of inflammatory cells 1 day post-injury, mainly mononuclear cells, which is restricted to the damaged region adjacent to the deep uninjured region. *Right:* TA muscle 3 days post injury. At this time point, the level of inflammatory cell infiltration (indicated) has peaked. For additional images of freeze injured muscle, please see Ref. 23

Fig. 4.

Loss and recovery of muscle strength in two female mice after freeze injury to the left TA muscles. Maximal isometric torque of the left anterior crural muscles was measured immediately before and after injury was induced and again at 3, 7, 14, 21, and 28 days post-injury in the same two mice. Both mice had recovered to 90 % or more of the pre-injury level by day 21